

## Polynucleotide

Present invention relates to a novel polynucleotide involved in heritable Parkinson's disease (PD), a novel polypeptide encoded by the polynucleotide, and a method for  
5 diagnosing heritable Parkinson's disease (PD).

## Background

Parkinsonism (MIM168600) is a clinical syndrome characterized by bradykinesia, resting tremor, muscle rigidity, and postural instability (Gelb et al. 1999). The most  
10 common cause of parkinsonism is Parkinson's disease (PD). Second to Alzheimer's disease, PD is the most common neurodegenerative disorder affecting >1% of the population over 55 years of age (de Rijk et al. 1995). Neuropathological findings in PD are loss of pigmented neurons in the brainstem, *substantia nigra* and *locus ceruleus*, with intracellular Lewy body inclusions found within surviving neurons (Forno 1996).

15 Although PD is considered a sporadic disease, various hereditary forms of parkinsonism have been recognized (Vila and Przedborski 2004). A major breakthrough in recent years has been the mapping and cloning of a number of genes causing monogenic forms of parkinsonism. Genomic multiplication and missense mutations in the  $\alpha$ -synuclein gene were initially identified in a small number of families with autosomal dominant  
20 parkinsonism (PARK1/4 [MIM 168601]) (Polymeropoulos et al. 1997; Kruger et al. 1998; Singleton et al. 2003; Chartier-Harlin et al. 2004; Farrer et al. 2004; Zarranz et al. 2004). Subsequently,  $\alpha$ -synuclein antibodies were found to robustly stain Lewy bodies and Lewy neurites in the *substantia nigra* in familial and sporadic PD (Spillantini et al. 1997) and common genetic variability in the  $\alpha$ -synuclein promoter has been implicated in sporadic PD  
25 (Pals et al. 2004).

Autosomal recessive mutations in three genes, *parkin*, *DJ-1* and *PINK1* have been linked with early-onset parkinsonism (<45 years at onset) (PARK2, PARK6 & PARK7 [MIM 602533, 602544 & 608309])(Kitada et al. 1998; Bonifati et al. 2003; Valente et al. 2004). A large number of pathogenic mutations and rearrangements have been identified in the  
30 *parkin* gene reviewed by (Mata et al. 2004), but mutations in DJ-1 and PINK-1 are rare (unpublished data).

Very recently, five pathogenic mutations were identified in a gene, leucine-rich repeat kinase 2 (*LRRK2*) in six families with autosomal-dominant parkinsonism, linked to the PARK8 locus [MIM 607060]) (Zimprich et al. 2004a). Paisan-Ruiz and colleagues

independently confirmed these findings of two pathogenic mutations in a British and Basque families (Paisan-Ruiz et al. x2004).

### Object

- 5       The object of the invention is to isolate a gene or polynucleotide proving inheritable parkinsonism, and to use presence of this gene to diagnose a patient before he/her gets sick. A further object is to use this gene or polynucleotide to transfect a microorganism or experimental animal in order to develop a new medicine for treating or preventing the onset of parkinsonism.

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### The invention

- Inheritable parkinsonism may be proved by the method according to the characterizing part of claim 5, and the other objects are met by a polynucleotid according to the characterizing part of claim 1, a recombinant vector according to claim 3, a DNA probe and  
15 a DNA primer according to claims 4 and 6 respectively, and a peptide according to claim 9.

- The inventors have isolated a novel LRRK2 mutation, and this mutation may cause development of dominantly inherited PD. By screening healthy persons, one can state whether the healthy persons have the mutation, and thus most likely will develop the  
20 illness.

- Using a probe to test whether a patient has the mutation allows a precise, differential diagnosis of this type of Parkinson's disease. The probe represents a safe and accurate biomarker which will be powerful as it nominates subjects, future patients, for neuroprotective therapy. At the present time this is a research enterprise, but not for  
25 long. These subjects provide the first (and only) 'uniform substrate/background' for studies on drug efficacy/safety. From a research perspective **they** will also facilitate models of disease (C.elegans, Drosophila, mice) and epidemiological research on the variable expressivity and age-associated penetrance. **As the sequence of the mutated gene is known, microorganisms and further experimental animals may be transfected, in**  
30 **order to investigate for a new medicine to treat or prevent the onset of the illness.**

The genetic information provides subjects with the cause of their disease, an explanation for which, if handled correctly, can be of great psychological benefit (fulfilling the 'need to know' why). This information also prioritizes the resources of the research community,

grant funding agencies and the pharmaceutical industry on developing a neuroprotective therapies to halt G2019S disease progression.

In the following the invention will be described by reference to a study of PD patients  
5 and their families. Parts of the study are shown in figures, wherein

Figure 1 shows a schematic drawing of LRRK2 with predicted protein domains,

Figure 2 shows pedigrees of families with LRRK2 G2019S,

Figure 3 shows chromosome 12q12 STR markers on the disease haplotype (PARK 8),

Figure 4 shows probability of becoming affected by parkinsonism, in LRRK2 G2019S  
10 carriers, as a function of age, and

Figure 5 shows aligned amino acid sequences of the activation loop of different human kinases.

The inventors identified seven unrelated persons all having the new mutation, from 248  
15 multiplex kindreds with dominantly inherited PD, and six further unrelated persons from three populaton-based series of persons with dominantly inhereted PD. These 13 persons and their families made basis for the inventors' further work. Segregation and linkage analysis provides evidence for pathogenicity and an estimate of age-associated penetrance; haplotype analysis demonstrates the mutation originates from a common and ancient  
20 founder.

## **Subjects and Methods**

### *Study subjects*

The patients and controls were examined by neurologists specialized in movement  
25 disorders. A full history, including family history and neurological examination, was completed on each patient. Clinical diagnosis of PD required the presence of at least two of three cardinal signs (resting tremor, bradykinesia and rigidity), improvement from adequate dopaminergic therapy and the absence of atypical features or other causes of parkinsonism.

### *LRRK2 sequencing and mutation screening*

Blood samples were taken and genomic DNA was extracted using standard techniques. Six families (families 194, 281, 3081, 3082, 3083 and 3211) were known to have a positive LOD-score for STR (Short Tandem Repeat) markers in the PARK8 locus (Zimprich et al.

2004b). Amplification of all 51 exons of the *LRRK2* gene was performed by polymerase chain reaction (PCR) in one patient having PD, from each of these six families. All PCRs were carried out for each primer set with 20-50 ng of template DNA in a total volume of 25µl using a final reaction concentration of 200 µM dNTP, 1x PCR-Buffer (Qiagen), 1x Q-  
 5 Solution (Qiagen), and 0.8 µM of each primer. One unit of Taq polymerase (Qiagen) was added to each reaction. Amplification was performed using a 57-52°C-touchdown protocol over 38 cycles. The primers used for PCR amplification of *LRRK2* exons and for sequencing are available on request.

The nucleotide sequences of all PCR products were determined by direct sequencing.  
 10 Each PCR product was cleaned by using a Millipore PCR purification plate. Three microliters of purified PCR product was used per sequencing reaction with 1 µl of either the forward or reverse PCR primer and 1 µl of BigDye reaction mix (Applied Biosystems). Electrophoresis was performed under standard conditions on an ABI 3730 automated sequencer (Applied Biosystems). All sequences were obtained with both forward and  
 15 reverse primers. Sequences were analyzed with SeqScape software version 2.1.1 (Applied Biosystems) and compared with published sequence of *LRRK2* (GenBank accession no. AY792511).

After identification of a heterozygous G2019S (G6055A) mutation in the proband of family 3215 (referred to as family 3211 in Zimprich et al, 2004b), we designed a probe  
 20 employing TaqMan chemistry on an ABI7900 (Applied Biosystems) to screen for this mutation. First we examined 248 PD patients from families with a known family history, consistent with autosomal dominant transmission of a suspected causative gene. Then 377 Norwegian, 271 Irish and 100 Polish PD patients (constituting the three population series) were checked using this assay; and 2260 samples of healthy persons from similar  
 25 populations were also included (1200 US American, 550 Norwegian, 330 Irish and 180 Polish subjects), the latter to be used as control samples. Mutations were confirmed by direct sequencing of PCR products from *LRRK2* exon 41. Finally, all participating family members of *LRRK2* G2019 mutation carriers (affected and unaffected) were screened for the mutation.

30 By 6055 G>A or G6055A it is meant that nucleotide number 6055 of the *LRRK2* gene, counted from the 5'end of the polynucleotide, has changed from G (guanine) to A (adenine). This change also causes a change in the polypeptide encoded by the polynucleotide, and G2019S denotes a polynucleotide where aminoacid number 2019 is

changed from G(Glycine) to S(Serine). These shortenings are wellknown to persons skilled of the art.

#### *Genotyping of STR markers*

- 5 Fourteen STR markers were genotyped in mutation carriers and all available family members, in all 13 families, for linkage analyses and to determine whether there was a particular haplotype associated with the *LRRK2* mutation. STR markers were chosen to span the PARK8 region including D12S87, D12S1648, D12S2080, D12S2194, D12S1048, D12S1301 and D12S1701. *LRRK2* is located between D12S2194 and D12S1048. We also
- 10 developed seven novel STR markers in this region (shown in table 1 below) by searching for repeat polymorphisms using RepeatMasker of *in silico* BAC sequence (UCSC Human Genome Browser Web site). The labeling of these novel markers reflects their physical position relative to the start codon of *LRRK2*.

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<b>Table 1. Novel chromosome 12 STR markers</b>		
Marker name	Primer sequence	Physical position (bp) On chromosome 12
D12S2514	F: 5'-TTGCAGCTGTAAGGAATTTGGG-3'	38873779
	R: 5'-GCATTCTTCAGCCTGAGACCC-3'	
D12S2515	F: 5'-TGAAGGACACTGAACAAGATGG-3'	38974140
	R: 5'-GCCATAGTCCTTCCATAGTTCC-3'	
D12S2516	F: 5'-CGCAGCGAGCATTGTACC-3'	38989214
	R: 5'-CTCGGAAAGTTTCCCAATTC-3'	
D12S2518	F: 5'-CTGGTATTACCTCAACTGTGGCTC-3'	39034800
	R: 5'-ACTGGTATGTTTAAGCCTGGCAC-3'	
D12S2519	F: 5'-AGCAGCAGAGAAGATTTCAATAAC-3'	39116816
	R: 5'-AATCATCTTTGAAAGAACCAGG-3'	
D12S2523	F: 5'-TAAACGAAGCTCCCTCACTGTAAG-3'	39147728
	R: 5'-TCTTTGTAGCTGCGGTTGTTC-3'	
D12S2517	F: 5'-TCATGAAGATGTCTGTGATAGGGC-3'	39282976
	R: 5'-CTCTATTGTGAGCAAACATGCATGG-3'	

- One primer of each pair was labeled with a fluorescent tag. PCR reactions were carried out on 10-20 ng of DNA in a total volume of 15  $\mu$ l with final reaction concentrations of 150  $\mu$ M dNTP, 1x PCR-Buffer (Qiagen), 1x Q-Solution (Qiagen) and 0.6  $\mu$ M of each primer, with 1 unit of Taq Polymerase (Qiagen). Amplification was performed using a 57-52°C-  
5 touchdown protocol over 38 cycles. The PCR product for each marker was diluted by a factor of 10 to 100 with water. One microliter was then added to 10  $\mu$ l of Hi-Di Formamide and Rox size standard. All samples were run on an ABI 3100 genetic analyzer, and results were analyzed using Genescan 3.7 and Genotyper 3.7 software (Applied Biosystems).  
10 Since population allele frequencies were not available from the CEPH database, these have been estimated by genotyping 95 unrelated Caucasian subjects, a population based series from the United States (shown in table 2 below).

Table 2. Allele frequencies of Park 8 Markers	
Marker and allele (bp)	Frequency (%)
D12S87 (n = 92)	
150	0.5
154	1.1
156	27.2
158	33.2
160	11.4
162	2.7
164	6.0
166	17.4
168	0.5
D12S1648 (n = 91)	
110	13.7
112	3.3
114	11.0
116	4.4
118	2.2
120	2.8
122	17.0
124	3.9
126	7.7
128	14.3
130	8.8
132	2.8
134	2.8
136	1.7
138	0.6
140	2.2
142	1.1
D12S2080 (n = 93)	

**Table 2. Allele frequencies of Park 8 Markers**

Marker and allele (bp)	Frequency (%)
176	1.6
180	20.2
184	44.7
188	22.9
192	10.6
D12S2194 (n = 87)	
245	0.6
249	40.9
253	32.4
257	19.9
261	4.6
265	1.7
D12S2514 (n = 82)	
284	11.0
291	53.1
294	32.3
297	1.2
300	2.4
D12S2515 (n = 93)	
208	3.2
212	26.6
216	18.6
220	22.9
224	20.7
228	5.3
232	2.7
rs 7966550 (n = 90)	
T	90.6
C	9.4
DS12S2516	
252	37.3
254	62.7
rs 1427263 (n = 89)	
A	63.6
C	36.5
rs1116013 (n = 88)	
A	49.4
G	50.6
rs11564148 (n = 88)	
A	26.1
T	73.9
D12S2518 (N = 90)	
154	79.7
168	15.9
170	4.4
D12S519 (n = 72)	
132	29.5

<b>Table 2. Allele frequencies of Park 8 Markers</b>	
<b>Marker and allele (bp)</b>	<b>Frequency (%)</b>
134	22.6
138	22.6
140	25.3
<b>D12S2520 (N = 85)</b>	
248	8.2
251	7.6
254	10.0
257	54.1
260	20.0
<b>D12S2521 (N = 93)</b>	
311	0.5
315	10.8
319	20.4
323	8.1
327	7.0
331	8.1
335	0.5
355	1.1
359	7.5
363	13.4
367	7.0
371	7.0
375	6.5
379	3.8
383	1.1
387	.5
<b>D12S2522 (N = 93)</b>	
281	9.1
283	14.0
285	.5
287	11.3
293	.5
295	15.6
297	44.6
299	4.3
<b>D12S2523 (n = 89)</b>	
305	18.9
314	41.1
317	8.9
320	30.0
323	1.1



Table 2. Allele frequencies of Park 8 Markers	
Marker and allele (bp)	Frequency (%)
D12S2517 (n = 93)	
180	8.5
182	7.5
184	15.4
186	8.5
188	11.7
190	8.0
192	5.3
194	1.1
196	1.1
198	3.2
200	0.5
202	3.7
204	6.9
206	6.9
208	4.3
210	2.1
212	3.2
214	1.6
216	0.5
D12S1048 (n = 89)	
211	37.2
214	21.1
217	17.8
220	2.2
223	6.7
226	11.7
229	3.3
D12S1301 (n = 93)	
96	0.5
100	37.2
104	17.6
108	11.1
112	12.2
116	13.3
120	7.5
124	0.5

**Table 2. Allele frequencies of Park 8 Markers**

Marker and allele (bp)	Frequency (%)
D12S1701 (n = 93)	
89	4.3
91	4.8
93	10.8
95	40.0
97	16.0
99	12.4
101	11.8
103	0.5

A The number of individuals genotyped is given for each marker (n)

B Alle frequencies are for individual markers in U.S. control subjects

## 5 *Statistical Analysis*

Multipoint nonparametric LOD scores for all families were calculated using GENEHUNTER-PLUS (Kong and Cox 1997). The frequency of the deleterious allele was set at 0.0001, and empirically determined allele frequencies were employed. The map positions for each marker were taken from Rutgers combined linkage-physical map version 1.0 (MAP-O-MAT web site). The three loci D12S2080, D12S2194 and D12S1301 are very tightly linked, with no observed recombinants in the database or within our genotyped families, and thus inter-marker distances were assigned as 0.01cM.

Chromosome 12 haplotypes in the PARK8 region were established for those families in which chromosome phase for mutation-carrying individuals could be deduced, thereby determining which alleles co-segregated with the *LRRK2* G2019S mutation in each family. For those affected individuals in whom the associated allele for a marker could not be determined, both alleles are given.

The age-dependent penetrance was estimated as the probability of a gene carrier becoming affected, at a given age, within the 13 families. The number of affected mutation carriers, for each decade, was divided by the total number of affected individuals, plus the number of unaffected carriers within that range. For some affected family members no DNA was available and only historical data on the disease course was obtained. These individuals were excluded from penetrance calculations.

## 25 **Results**

As mentioned previously, we identified 13 affected probands (i.e. 13 patients) who carry a heterozygous G6055A mutation in exon 41 of the *LRRK2* gene. The mutation leads

to a G2019S amino acid substitution of a highly conserved residue within the predicted activation loop of the MAPKKK (Mitogen-Activated Protein Kinase Kinase Kinase) domain (figure 1). After genotyping a total of 42 additional family members, 22 additional subjects were found to carry the mutation, seven with a diagnosis of PD (shown in table 3 below). One affected member of family P-089 did not carry the mutation and, for the purposes of this study, was considered a phenocopy and excluded from further analyses. Seven families originated from Norway, three were from the United States, two from Ireland, and one was from Poland. One family from the United States descended from Russian/Rumania, and another from Italy. For only one family (family 111), the ethnic origin was unknown. The *LRRK2* G2019S mutation segregates with disease in all kindreds, consistent with autosomal dominant transmission. To ensure patient confidentiality, simplified versions of the family pedigrees are presented in figure 2. There was no evidence of the mutation in the 2260 control samples.

Age at onset of clinical symptoms was quite variable, even within the same family. Family 1120, a family from the United States, had both the earliest and latest age at onset for a patient. The youngest affected subject had an onset at 39 years, whereas the oldest carrier presented with initial symptoms at 78 years. Where recorded, most *LRRK2* G2019S carriers have late-onset disease (>50 years at onset). The mean age at onset of affected mutation carriers was 56.8 years (range 39-78 years, n=19). Unaffected carriers have a mean age of 53.9 years (range 26-74 years, n=14). The penetrance of the mutation was found to be highly age-dependent, increasing from 17% at the age of 50 to 85% at the age of 70 (figure 4).

Table 3													
Demographic and Clinical Information for 13 Families with LRRK2 G2019S													
CHARACTERISTIC	Country of origin	FINDINGS FOR FAMILY											
		P-063 Norway	P-089 Norway	P-104 Norway	P-241 Norway	P-369 Norway	P-394 Norway	F05 Norway	1210 United States	111 United States	1120 United States	PD66 Ireland	3211 Ireland
No. of generations		3	4	3	3	3	4	4	2	2	3	1	2
No. of affected individuals		2	4	4	1	3	4	5	2	3	3	1	3
No. of typed individuals affected (unaffected)		1 (6)	2 (8)	1 (1)	1 (4)	2 (3)	1 (1)	3 (6)	1 (0)	2 (0)	3 (3)	1 (0)	2 (6)
No. of typed generations		2	3	1	2	1	2	2	1	1	2	1	1
Age <sup>a</sup> at onset in years (range)		59 (53-65)	59 (43-70)	58	60	50 (43-61)	66 (61-70)	64	65	58 (57-58)	59 (39-78)	41 (40-52)	46
Maximum mLOD score		0	.30	0	0	.60	0	.90	0	.09	.30	0	.30
<sup>a</sup> Average ages at onset are given when affected individuals. $n \geq 2$													
													IP Poland

Evidence for linkage (the statistical burden of proof that this mutation causes disease) to the PARK8 locus was found across families, with a combined maximum multipoint LOD score of 2.41 [for all 14 markers], corresponding to a P value of  $4.3 \times 10^{-4}$ . As only a defined chromosomal region was investigated, rather than a genome-wide search, this LOD score exceeds that required for significance,  $P=0.01$  (Lander and Kruglyak 1995). A positive LOD score was found in all families where more than one affected subject was genotyped (table 3).

All affected members from the different families, except the individual in family P-089 who did not carry the mutation, appear to share a common haplotype on chromosome 12 the *LRRK2* gene locus (figure 3). Haplotypes can be established with certainty in nine of the families, and all mutation carriers in these families share alleles for four STR markers and 4 single nucleotide polymorphisms (SNPs) in the *LRRK2* gene locus. These markers are *LRRK2* D12S2516, D12S2518, D12S2519, D12S2520 and SNPs rs7966550, rs1427263, rs11176013, rs11564148. For the remaining families, the number of available samples from relatives was not sufficient to determine phase. However, the genotypes in these cases are consistent with a common *LRRK2* G2019S allele. D12S2516 is located in intron 29 and D12S2518 is located in intron 44 of the *LRRK2* gene, whereas the two other shared markers are positioned 3' of the gene. Using the physical position of the shared and non-shared markers, the size of the shared haplotype is between 145 kb and 154 kb.

### Discussion

We have identified a novel *LRRK2* mutation, G2019S, which co-segregates with autosomal dominant parkinsonism in 13 kindreds originating from several European populations. Positive LOD scores were obtained in multiplex families, and combined they provide significant support for the PARK8 locus. *LRRK2* G2019S mutation was absent in a large number of control subjects, and of similar ethnicity. The number of families linked to *LRRK2* in this and previous studies now explains the majority of genetically defined autosomal dominant parkinsonism.

The mean age at onset of affected *LRRK2* G2019S carriers was 56.8 years, and comparable to that of patients in other families linked to PARK8 (Funayama et al. 2002; Paisan-Ruiz et al. 2004; Zimprich et al. 2004a). The majority of patients present with late-onset disease, indistinguishable from typical idiopathic PD. Disease penetrance is age-dependent, and increases in a linear fashion from 17% at the age of 50 to 85% at the age of 70. Age is the single most consistent risk factor for development of PD and other neurodegenerative disorders (Lang and Lozano 1998), and an important risk factor in *LRRK2* associated parkinsonism. Interestingly, age at onset was variable in this study, both within and between different families, suggesting other susceptibility factors, environmental or genetic, may influence the phenotype.

Although our findings clearly indicate that *LRRK2* mutations account for a substantial proportion of familial late-onset parkinsonism, historically, cross-sectional twin studies have not supported a genetic etiology for late-onset PD (Tanner et al. 1999; Wirdefeldt et

al. 2004). The age-associated penetrance of *LRRK2* mutations provides some explanation as even large and well designed twin studies are underpowered to detect incompletely penetrant mutations (Simon et al. 2002). *LRRK2* mutations were also found in apparently sporadic PD patients; three of the patients in this study did not have any known affected  
 5 first- or second-degree relatives. However, a caveat of age-dependent penetrance is that carriers may die of other diseases, before manifesting or being diagnosed with PD. Thus, it seems difficult to separate sporadic and familial PD, or to hypothesize environmental causes to be more important in one group and genetic causes more prominent in the other. In light of these results, a family history of parkinsonism, previously considered an  
 10 exclusion criterion for a diagnosis of PD, must be reconsidered (Hughes et al. 1992).

*LRRK2* is a member of the recently defined ROCO protein family (Bosgraaf and Van Haastert 2003). In human, mouse and rat, members of the ROCO protein family have five conserved domains (figure 1). The kinase domain belongs to the MAPKKK subfamily of kinases. The active sites of all kinases are located in a cleft between an N-terminal and a C-  
 15 terminal lobe, typically covered by an 'activation loop', in an inactive conformation. The activation loop must undergo crucial structural changes to allow access to peptide substrates and to orientate key catalytic amino acids (Huse and Kuriyan 2002). In different kinases, the activation loop starts and ends with the conserved residues asp-phe-gly (DFG) and ala-pro-glu (APE), respectively (Dibb et al. 2004). Of note, the *LRRK2* G2019S  
 20 substitution changes a highly conserved amino acid at the start of this loop (figure 5). In a German family we previously described, an I2020T mutation is located in an adjacent codon (Zimprich et al. 2004a). In other kinases, oncogenic mutations in residues within the activation loop of the kinase domain have an activating effect (Davies et al. 2002), thus we postulate *LRRK2* G2019S and I2020T mutations may have an effect on its kinase activity.

25 The age of an allele may be estimated from the genetic variation among different copies (intra-allelic variation), or from its frequency (Slatkin and Rannala 2000). However, the local recombination rate on chromosome 12q12 is unknown, as is the frequency of the G2019S mutation in the general population. Nevertheless, at centromeres there is generally a dearth in recombination; indeed no crossovers have been observed between *LRRK2*  
 30 flanking markers D12S2194 and D12S1048 in our studies, or within CEPH families (MAP-O-MAT web site). The physical size of the shared haplotype is also small, between 145 kb and 154 kb, and the allele is widespread in families from several European populations. Hence, the mutation is likely to be ancient and may be relatively common in specific

populations. These data suggest a substantial proportion of late-onset PD will have a genetic basis.

## 5 Electronic-Database Information

The physical position of markers is from NCBI build 34. Accession numbers and URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

10 MAP-O-MAT, <http://compgen.rutgers.edu/mapomat>

RepeatMasker, <http://www.repeatmasker.org/>

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